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ANAPHYLAXIS REACTIONS WITH PURIFIED PROTEINS FROM MILK *

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In the course of a study of the proteins of cow's milk by one of us (O),[1] four proteins were isolated in a high degree of purity, and their individuality established by means of the anaphylactic reaction. Evidence of the essentially chemical basis of immunologic specificity, as emphasized in our previous communications on the immunologic reactions of the vegetable proteins,[2] thus received further support. Our experiments were performed chiefly to determine the chemical individuality of the various protein preparations under investigation, and the completeness of their separation from one another. However, since these results had an important bearing on problems of immunologic specificity, numerous additional experiments have been made from this standpoint with these carefully isolated proteins.

As one of the most readily available protein-containing solutions, milk perhaps has been used in immunologic experiments with greater frequency than any other material except blood and egg white. The ease with which casein can be separated from milk in a relatively pure condition and its ready solubility in dilute alkali have made this protein particularly suitable for use when a pure isolated protein was desired. Hence many references are to be found in the literature dealing with the immunologic reactions of casein. It is evident, however, from the published accounts, that the casein used in much of this work was not so carefully purified as it should have been. Furthermore, in only a few instances in which the proteins of the whey [3] have been used, have these been separated from each other with the care necessary to secure sufficiently pure preparations. As we have found no complete review of the literature of the immunologic reactions of the several proteins of milk, we here present a brief synopsis in approximately chronologic order.

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[1] Osborne and Wakeman: Jour. Biol. Chem., 1918, 33, pp. 7 and 243.

[2] Wells and Osborne: Jour. Infect. Dis., 1913, 12, p. 341; 1916, 19, p. 183.

[3] The term whey is here used to designate the solution of the milk solids from which casein has been removed by precipitation with acid.

IMMUNOLOGIC EXPERIMENTS WITH MILK AND MILK PROTEINS

Although the earliest experimental work on immunologic reactions with protein solutions dates to 1897, apparently isolated casein was not used until 1901. Moro,[4] Hamburger,[5] and Levene[6] independently, and almost simultaneously, published observations on the precipitin reactions with casein. Moro reported that by immunizing with pure casein, from either human or cow's milk, he obtained a "specific lactoserum," but he did not describe its properties further. He also reported that precipitins for cow's milk gave reactions with goat's milk, but not with human milk, which was not in agreement with the earlier work on milk precipitins by Wassermann and Schütze.[7] The important observation was made that heating milk one-half hour at 100 degrees did not destroy its reactivity or its antigenic property.

Hamburger[5] found that "lactalbumin" (which included all the proteins in solution after removing the casein) could be distinguished from casein by the precipitin reaction. Antilactalbumin serum (or, more properly, antiwhey serum) gave reactions with whole milk and with lactalbumin, but not with casein, and anticasein serum reacted with whole milk and casein, but not with whey. He prepared his casein by precipitating with acetic acid and then washing the precipitate free from "albumin." He also used Schlossmann's method of filtering off the casein through a "Thonzellenfilter," and stated that the "albumin" in the filtrate behaved somewhat differently from that obtained by precipitating out the casein.

In connection with other work, Levene[6] merely reported that immune serum for milk gave precipitates with milk, casein, "milk albumin" and beef serum.

Fuld[8] stated that whey obtained by filtering milk through porcelain filters did not react with antimilk serum. Since this is not in harmony with observations by others, it probably means that his immunization was sufficient to produce precipitins only for casein, and not for the other less abundant milk proteins. His statement that heated milk or casein solution did not produce precipitins has been shown to be incorrect. His antiserum for cow's milk did not react with goat's milk or with isolated casein from human milk.

Müller,[9] in a study of the relation of specific antiserum precipitins to rennin, observed that antimilk serum did not precipitate casein except in the presence of calcium salts, although it united with the casein in their absence. He found no evidence that the precipitation of casein by antiserum was accompanied by the splitting off of proteins similar to those of whey. In another article[10] he stated that the products of digestion of casein with pepsin or trypsin did not produce precipitins either for themselves or for casein, although paracasein and iodocasein immunization gave precipitins that precipitated casein. Lactoserum did not precipitate paracasein.

Gengou[11] isolated casein, lactalbumin and lactoglobulin from cow's milk, finding that the casein and globulin gave complement fixation and precipitin reactions with antimilk serum, but the albumin did not react. The antiserum for cow's milk reacted with goat's and sheep's milk, and less strongly with human and horse's milk.

[4] Wien. klin. Wchnschr., 1901, 14, p. 1073.

[5] Ibid., 1901, 14, p. 1202.

[6] Med. News, 1901, 79, p. 981.

[7] Verein f. inn. Med., Verinsbeilage, July 26, 1900, p. 178; Schütze. Ztschr. Hyg. u Infektionskr., 1901, 36, p. 5.

[8] Hofmeister's Beitr., 1902, 2, p. 425.

[9] Arch. f. Hyg., 1902, 44, p. 126.

[10] Centralbl. f. Bakteriol., I. O., 1902, 32, p. 521.

[11] Ann. de Inst. Pasteur, 1902, 16, p. 734.

Schlossmann and Moro,[12] who discussed the methods used in the separation of milk proteins, recognized that the so-called milk "albumin" is a mixture of globulin and albumin, containing no phosphorus, which statement later studies have shown to be incorrect as far as globulin is concerned. They found also that the casein and albumin fractions each gave specific reactions for the species from which they were derived, and also that globulin from human serum reacted with antihuman-milk serum, but not with anticow's-milk serum. Antiserum for human milk gave precipitates with human serum.

Amberg,[13] who reprecipitated his preparations, found that the precipitin reaction with milk or casein did not depend on the presence of either inorganic or casein salts of calcium, thus contradicting Müller. He observed no differences between the reaction with anticasein and antimilk serums when used against casein, milk, or lactalbumin.

Fleischer[14] immunized animals with purified casein from animals of various species (rabbit, cat, dog, horse, goat, cow and human), and found that the antiserums gave precipitin reactions with both the homologous and foreign caseins, although with quantitative differences in favor of the homologous caseins. Antiserum for cow's casein gave about two-thirds as much precipitate with goat's casein as with cow's casein, and a good precipitate was obtained with horse's casein, but only a trace with dog's, cat's and rabbit's casein, and none with human casein. Antiserum for human casein gave only relatively slight reactions with all the other caseins.

In their early studies of anaphylaxis, Rosenau and Anderson[15] made some experiments with milk, obtaining no reactions when cow's milk was injected into guinea-pigs that had been sensitized with either human or dog's milk, but positive results were given when the sensitization had been caused by sheep's milk. The first examination of isolated casein by means of the anaphylaxis reaction is reported by Besredka,[16] who stated that casein gave the same results as milk. The whey was found to sensitize to milk sometimes, but it did not always give reactions in animals sensitized to milk. Perhaps the difficulty here lies in the use of too small amounts of the whey (1/20 to 1/4 c c), which has a very small protein content.

Wells[17] observed that purified casein was even more active in anaphylactic reactions than whole milk, for 0.1 to 0.25 gm. of purified cow's casein usually produced fatal reactions when injected into the peritoneum of sensitized guinea-pigs, while doses of 5 to 10 c c of whole milk containing 0.15 to 0.30 gm. of casein were seldom followed by fatal reactions. The activity of casein solutions was not materially impaired by heating at 100 degrees for 25 minutes. Animals sensitized to goat's milk casein reacted severely when given cow's casein, or conversely, which agrees with Bordet's results with whole milk, and Fleischer's precipitin reactions with goat's and cow's casein.

A related observation on the lack of species specificity with casein is furnished by Michaelis and Rona,[18] who injected cow's casein subcutaneously into dogs and guinea-pigs that had recently been actively secreting milk, and observed a notable swelling of the mammary glands, without marked constitutional symptoms. Some reaction was obtained in virgin animals. This they interpret as the production of a heightened secretory activity which induced

[12] München. med. Wchnschr., 1903, 50, p. 597.
[13] Jour. Med. Research, 1904, 12, p. 341.
[14] Roussky Wratsch., 1908, 7, p. 1638.
[15] Jour. Med. Research., 1907, 16, p. 391.
[16] Ann. Inst. Pasteur, 1909, 23, p. 166.
[17] Jour. Infect. Dis., 1908, 5, p. 480; 1911, 9, p. 147.
[18] Pflüger's Arch., 1909, 121, p. 163.

the mammary glands to excrete the injected casein, although in none of the animals did actual secretion of milk follow the injection. On the other hand, Felländer[18a] was unable to sensitize guinea-pigs to guinea-pig's milk or to extracts of their mammary glands.

Bachrach[19] failed to secure a good differentiation between casein, whey and beef serum by anaphylactic reactions. As he used the same sized doses for both sensitizing and intoxicating, his observations are not valuable.

The use of the complement fixation reaction with casein was introduced by Kollmeyer,[20] who obtained distinct differentiation of casein from whey by this method.

Graetz[21] studied particularly the relation of milk to colostrum, and the only feature of his work of interest in this connection is the evidence obtained that colostrum whey contains proteins which seem to be identical with those of the serum. Other workers have obtained evidence of similar significance, indicating the whey proteins to be closely related to the serum proteins. Thus, Bauer[22] concluded that casein can be differentiated by complement fixation from the protein of the whey; that casein of different species of animals gives common group reactions, as also do the whey proteins, but that only the casein can be differentiated from the serum proteins of the same species. Bauereisen[23] finds that the complement-fixation method, like the precipitin reaction, does not permit an absolute differentiation of milk proteins from other proteins of the same individual, but that both methods show casein to be much less closely related to the serum proteins than are serum proteins to whey proteins. He also recognized the probability that the failure to distinguish sharply between the isolated proteins depends on defects in the method of their preparation.

The quantitative differentiation of milk proteins demonstrable by complement fixation is great enough to be recognized by the less sensitive anaphylaxis reactions, as indicated by the results obtained by Kleinschmidt.[24] He separated casein, globulin and albumin from cow's milk, but in his description of the process used does not indicate that he reprecipitated his preparations. His "casein" was everything that came down with sufficient acetic acid; the "globulin" was the precipitate produced by saturating with magnesium sulfate after removing the casein with NaCl, and the "albumin" was the precipitate obtained by adding acetic acid to the filtrate from the globulin. He found that anaphylaxis reactions (intracardiac) gave distinct differentiation between these three proteins. Casein sensitized to itself as well as whole milk, but did not sensitize to beef serum, lactalbumin or lactoglobulin. Experiments in the reverse order were almost equally specific. There were some slight crossed reactions, especially between globulin and casein, and a distinct protection (anti-anaphylaxis) reaction in all cases, probably due to incomplete separation of the proteins used. Guinea-pigs sensitized with cow's casein did not react with human casein; no other foreign caseins were tried. Animals sensitized with either cow's lactalbumin or lactoglobulin reacted with bovine serum, and conversely; those sensitized with lactalbumin reacted with lactoglobulin, and conversely. These last results do not warrant the statement made

[18a] Ref. in Ztschr. f. Immunität., Ref., 1912, 6, p. 851.
[19] Vierteljahr. gericht. Med., 1910, 40, p. 235.
[20] Ztschr. f. Biol., 1910, 54, p. 64.
[21] Ztschr. f. Immunität., 1911, 9, p. 677.
[22] Berl. klin. Wchnschr., 1910, 47, p. 830.
[23] Ztschr. f. Immunität., 1911, 10, p. 306.
[24] Monatschr. f. Kinderheilk., 1911, 10, p. 402.

in the author's summary: "In anaphylaxis experiments the proteins of cow's milk, casein, albumin and globulin, can be differentiated from one another." There are quantitative differences in his reactions, to be sure, but those are not constant. It is quite evident that the author was working with imperfectly purified protein preparations. A similar difficulty is found in the work of Heuner[25] who used commercial (Merck) cow's milk albumin and casein, as well as a milk albumin and a globulin prepared by a chemist for him, the description not indicating that his preparations were thoroughly purified. Nevertheless, he also reached the conclusion that globulin and albumin of milk are distinct from casein, and that colostrum and mastitis milk are more nearly related to the blood serum antigens than is normal milk. The differentiation was less marked in anaphylaxis experiments than with complement fixation.

Bauer and St. Engel[26] separated casein, globulin and albumin in the same way as Kleinschmidt, but reprecipitated the globulin three times, although the albumin seems not to have been reprecipitated. Using the complement-fixation reaction, they found distinct quantitative differences between all three proteins. Thus, with an anticasein (bovine) serum, complement fixation was complete, or nearly so, in the following dilutions of 0.5% solutions: casein, 1:256; globulin, 1:32; albumin, 1:16; milk, 1:64. With antiglobulin serum the figures were: casein: 1:8; albumin, 1:32; globulin, 1:16,384. With antialbumin serum the figures were: casein, 1:1; globulin, 1:8; albumin, 1:64. Similar results were obtained with the globulin or casein of human milk. The proteins of one species were not tested against the antiserums for the other species. Cow's milk globulin could not be differentiated from beef serum globulin; hence these authors believed the globulin and albumin of milk or colostrum or serum to be identical.

Klein[27] made a study of the nature of casein antiserum by the precipitin test, finding evidence that there are in such an antiserum two precipitins. One acts only in the presence of $CaCl_2$, while the other acts best in the absence of calcium. Hence the presence of two different antigen groups is postulated in the casein molecule. So far as we can learn, this important work has not been reinvestigated. The suggestion of the existence of two antigenic groups in a purified protein is, however, in harmony with the observations of Wells and Osborne[28] with purified alcohol-soluble proteins from wheat, rye and barley.

In agreement with the results of Bauer and St. Engel, it was found by Kudicke and Sachs[29] that under suitable conditions antimilk serum will, by virtue of the whey proteins, give complement-fixation reactions with blood serum of the same species, but if milk is boiled, the antiserum it engenders will react with milk but not with blood serum. (Evidently this depends on the coagulability of the whey and serum proteins and the incoagulability of casein.) Antiserum for bovine serum will give slight reactions with cow's milk boiled for not over ten minutes. Uhlenhuth and Haendel[30] had previously found that boiled milk gave anaphylactic reactions only in animals sensitized with milk, whereas raw milk caused reactions in animals sensitized with either milk or serum of the same species.

[25] Arch. f. Kinderheilk., 1911, 56, p. 358.
[26] Biochem. Ztschr., 1911, 31, p. 46.
[27] Folia microbiol., 1912, 1, p. 101.
[28] Jour. Infect. Dis., 1913, 12, p. 341.
[29] Ztschr. f. Immunität., 1914, 20, p. 316.
[30] Ibid., 1910, 4, p. 761.

Versell[31] separated casein by adding acetic acid just short of precipitation, and then saturating the milk with CO_2; the precipitate was washed five times by centrifugation. The whey proteins were not separated from one another. Using the complement-fixation reaction, he found that antiserum for cow's milk reacted with goat's milk, but less strongly than with cow's milk, and more strongly than with human milk; the difference between the caseins of the three species was much less, and most between the wheys. Human milk antiserum gave reactions with human milk diluted to 1 : 31,250, with cow's milk at 1 : 1,250, with goat's milk at 1 : 50. Antiserums for milk or whey gave slight reactions with the serum of the same animal, but anticasein serum did not, as observed by Bauer and St. Engel, and Kudicke and Sachs; also heated casein was still antigenic but heated whey was not. Whey proteins showed the same degree of species specificity as blood serum, but casein showed much less species specificity, being rather in the class of the organ-specific proteins. He found the thermo-resistance of casein is only relative, for it is inactivated if heated sufficiently long or high.

Gay and Robertson[32] found that isolated casein, as well as the "paranuclein" derived from it by peptic digestion, were both distinctly antigenic, one sensitizing against the other. Immunization with casein produced much more active precipitating and complement-fixing antiserums than did paranuclein, and these antiserums also reacted much more strongly with casein than with paranuclein. That is, partial peptic digestion reduced but did not destroy the antigenic power of casein. Completely digested casein was not antigenic. When united with protamine, it retained its antigenic power, the resulting antiserum reacting with either casein or protamine caseinate.[33] These authors believed that the paranuclein synthesized by the "reverse action" of pepsin on the products of peptic digestion of casein is an effective antigen, sensitizing and immunizing (complement fixation) both to itself and the paranuclein produced by partial peptic digestion, and thought that they had thus established the identity of the materials and also that synthesis of a true antigenic protein had been accomplished.[34]

The statement that casein is not an active antigen, made by Dale and Hartley,[35] is supported by the fact that injection of milk itself has been used extensively in nonspecific foreign protein therapy, ordinarily without harmful results, and Müller[36] states that in an extensive experience he has never observed serious symptoms. However, the next article in the same journal, by Lubiner,[37] describes a nearly fatal reaction following intramuscular injection of 10 c c of milk into a girl, 8 days after the last of 3 daily injections. Oppenheim[38] also observed a similar case of severe reaction from milk. Of some interest may be the observation that the serum of parturient and lactating women produces a stronger positive Abderhalden reaction with casein than does the serum of men or normal or pregnant women (Kastan[39]). Also we may mention that milk has hemolytic properties, as first shown by Bertarelli.

[31] Ibid., 1915, 24, p. 267.
[32] Jour. Exper. Med., 1912, 16, p. 470.
[33] Gay and Robertson: Ibid., 1912, 16, p. 479.
[34] Ibid., Jour. Biol. Chem., 1912, 12, p. 233.
[35] Biochem. Jour., 1916, 10, p. 431.
[36] Deutsch. med. Wchnschr., 1918, 44, p. 545.
[37] Ibid., p. 547.
[38] Wien. klin. Wchnschr., 1917, 30, p. 1519.
[39] München. med. Wchnschr., 1914, 61, p. 2126.

Pfaundler and Moro found that it contains hemolytic complement. Bauer, Kopf and Sassenhagen observed that this is especially abundant in colostrum, which was corroborated by Schmidt (lit.).[40]

Taken all together, the results obtained by the several observers quoted seem to point definitely to the following conclusions:

1. Biologically, casein is quite as distinct from the whey proteins and the serum proteins as it is chemically.

2. The biologic reactions and chemical composition of caseins from different species of animals show close relationships.

3. The whey proteins as a whole are biologically similar to the soluble serum proteins of the animals from which they are derived. Colostrum contains a larger proportion of proteins of this type.

4. Casein from the milk of an animal of any given species shows a closer biologic relationship to the casein of another species than it does to either the whey proteins or to the serum proteins of its own species; the same is true of the chemical relations.

It is evident, however, that the amount of work done with proteins reasonably well isolated is as yet small, and in need of amplification and control.

THE CHEMISTRY OF MILK PROTEINS

In recent years the proteins of milk have received more careful study than previously, and several new facts must receive consideration in biologic work. Applying D. D. Van Slyke's amino nitrogen method, Crowther and Raistrick[41] found that from the chemical standpoint casein, lactoglobulin and lactalbumin were sharply differentiated and distinct proteins, and that they have the same composition whether prepared from colostrum or from milk. Only 0.03% of true globulin was found in milk; it was much more abundant in colostrum. As far as could be determined, the proteins designated as eulactoglobulin and pseudolactoglobulin are chemically identical. Lactoglobulin showed an amino-acid make-up similar to that of globulin from ox-blood, but marked differences were found between lactalbumin and serum albumin. These observations correspond with the results of the immunologic demonstration that casein is distinct from the whey proteins, and that the latter possess features common to the serum proteins of the same species. A series of milkings made twice daily after calving were analyzed, the average composition of the milk from 7 cows showing a steady drop in content of protein between the first and eighth milkings, the percentage of nitrogen present in the milk being shown in table 1. These figures indicate the relatively great abundance of globulin in colostrum, and its rapid reduction within the first 48 hours of lactation.

While caseins from different species do not show demonstrable quantitative or qualitative chemical differences by ordinary methods, a study of the products of racemization by Dakin's method yielded to Dudley and Woodman[42]

[40] Arch. f. Kinderheilk., 1911, 56, p. 342.
[41] Biochem. Jour., 1916, 10, p. 438.
[42] Ibid., 1915, 9, p. 97.

evidence of some structural differences. Caseins from sheep's and cow's milk consist of identical amino-acids, apparently in identical proportions, but when racemized the sheep's casein showed all the tyrosine and most of the lysine unracemized, while both were completely racemized in cow's casein.

Dale and Hartley[35] state that caseins from different species show no clear disparity of antigenic properties, but it is possible that finer quantitative methods will disclose distinct discrepancies, as was found to be the case with the albumins from hen's eggs and duck's eggs. These same authors state that the antigenic properties of casein are relatively feeble.

Osborne and Wakeman[43] found that the protein called lactoglobulin differed much in its solubilities from other globulins, and that it contained 0.24% of phosphorus, in which respect it resembled ovovitellin. In addition they obtained a small quantity of material that seemed to be a proteose, although not enough was obtained to permit positive identification. Of particular interest is the discovery that a protein is present in small amount in milk, which is readily soluble in 50-70% alcohol. This alcohol-soluble milk protein in its content of amino-acids is distinctly different in composition from casein.

We, therefore, have chemical evidence that milk contains at least 4 chemically distinguishable proteins: (1) casein, characterized by a high content of phosphorus: (2) lactalbumin, a water-soluble protein which contains no phosphorus; (3) lactoglobulin, which contains 0.24% of phosphorus; and (4) the alcohol-soluble protein recently described by Osborne and Wakeman.

TABLE 1

PERCENTAGE OF NITROGEN IN MILK

	Total	Casein	Albumin	Globulin	Nonprotein
1st milking	2.40	0.75	0.14	1.32	0.19
4th milking	2.40	0.51	0.11	0.31	0.04
8th milking	0.65	0.46	0.05	0.12	0.02

EXPERIMENTAL PART

Before describing our results we would first call attention to the fact that anaphylaxis experiments should be performed only with animals that have been raised on a known dietary which does not contain the proteins under investigation, and the same should be true of the mothers, since a certain amount of active immunization may result from the food proteins, which can be passively transferred to the fetus. It has been shown by one of us[44] that young guinea-pigs fed on a given protein (egg white or milk) after a short time become sensitized to that protein so that they will give typical reactions when it is injected

[43] Jour. Biol. Chem., 1918, 33, p. 7.

[44] Wells, H. G.: Jour. Infect. Dis., 1911, 9, p. 147.

parenterally. If the feeding is long continued the animals become so immunized after a time that even a sensitizing injection of this protein will not render them hypersensitive, and a second injection has no effect. Guinea-pigs raised largely on oats, therefore, do not give good

TABLE 2

CASEIN

	Sensitizing Dose, Gm.	Intoxicating Dose. Gm.	Results	Remarks
	Casein	Casein		
1	0.001	0.05	Died, 5 minutes	
2	0.001	0.05	Died, 15 minutes	
3	0.050	0.05	Severe	Note large sensitizing dose
4	0.050	0.05	Severe	
5	0.005	0.001	Slight, doubtful	
6	0.005	0.005	Slight	
7	0.005	0.010	Died, 60 minutes	
8	0.005	0.020	Severe	Nearly died
9	0.005	0.000,1	Died, 2 minutes	Intracardiac
10	0.005	0.000,02	Moderate	Intracardiac
11	0.005	0.000,01	Doubtful	Intracardiac
12	0.005	0.000,01	Slight, moderate	Intracardiac
13	0.001	0.100	0 ?	
14	0.001	0.100	Moderate	
15	0.000,5	0.100	Severe	Nearly died
16	0.000,5	0.100	0	
17	0.000,1	0.100	Slight	
18	0.000,1	0.100	0	
19	0.000,05	0.100	0	
	Casein	Lactalbumin		
20	0.050	0.100	Slight	
21	0.005	0.050	Slight	
22	0.005	0.050	0	
	Lactalbumin	Casein		
23	0.005	0.050	0	
24	0.005	0.050	0	
	Casein	Lactoglobulin		
25	0.005	0.050	0	
26	0.005	0.050	Doubtful	
	Lactoglobulin	Casein		
27	0.005	0.050	0	
28	0.005	0.050	Slight	
	Casein	Alcohol soluble protein		
29	0.001	0.050	Slight	
30	0.001	0.050	0	
31	0.005	0.050	0	
32	0.005	0.050	Moderate	
	Alcohol soluble protein	Casein		
33	0.005	0.050	Moderate	No protection
34	0.005	0.050	Severe	No protection
35	0.005	0.050	0	No protection
36	0.005	0.050	0	No protection
37	0.005	0.050	Died, 10 minutes	
38	0.005	0.050	Slight	
	Casein	Beef serum		
39	0.005	1.0 c c	0	
40	0.005	1.0 c c	0	
	Beef serum	Casein		
41	0.1 c c	0.050	0	
42	0.1 c c	0.050	0	

reactions with oat proteins, although readily sensitized to other, unrelated vegetable proteins; and guinea-pigs raised on corn were found not to serve for reactions with proteins from corn, although readily sensitized to oat proteins. Probably this fact accounts for numerous

failures and irrational results obtained in anaphylaxis work, and must be taken into account, particularly in experiments with proteins such as those of milk, that might be present in the guinea-pig dietary. In these experiments all our guinea-pigs have been raised exclusively on a vegetable diet, as have their mothers.

In determining specificity it is also imperative that every precaution be taken to avoid contamination, especially of the sensitizing doses, with even the smallest possible quantity of the protein that is to be used for the intoxicating dose. As numerous articles discussing anaphylactic specificity do not mention these precautions, leaving the reader uncertain as to the significance of the results described, we state explicitly that in this, as in all our work, such contaminations were rigidly excluded. All glassware, after thorough washing, is kept for at least 24 hours in concentrated sulphuric acid and potassium bichromate; the needles are most thoroughly cleaned and kept in absolute alcohol.

During the course of this work several preparations of various fractions, obtained in the isolation and purification of the milk proteins, were tested by means of the anaphylaxis reaction. The following tables give the results obtained with purified preparations of the 4 known proteins. The terms used are as defined in our previous work.[45] Unless otherwise specified the injections were made by the intraperitoneal route. The interval between sensitizing and intoxicating injections was usually about from 18 to 20 days. The references to protection indicate the protection of a sensitized animal against reinjection with the homologous antigen after previous injection of some heterologous antigen.

The first nineteen experiments recorded in this table deal with the antigenic properties of casein. They show that, on the whole, casein is not so active as some other soluble proteins in producing anaphylactic sensitization and shock, but, on the other hand, its activity is by no means low. By intracardiac injection the minimum lethal dose is apparently between 0.000,02 and 0.00001 gram, but by intraperitoneal injection it requires nearer 0.010 gram to cause death, and 0.020 gram is not always fatal. Presumably this great difference in the efficiency of intracardiac and intraperitoneal injections of casein depends on the readiness with which casein is thrown out of solution, for with the vegetable proteins we have found that their intraperitoneal toxicity for sensitized animals seems to vary directly with their solubility in the body fluids.[46] As compared with its intoxicating power the sensitizing

[45] Wells, H. G., and Osborne, T. B.: Jour. Infect Dis, 1911, 8, p. 88.
[46] Wells, H. G., and Osborne, T. B.: Jour. Infect Dis., 1914, 14, p. 377.

capacity of casein seems to be very low. Table 3 offers a comparison of the anaphylactogenic activity of the milk proteins with that of other proteins as determined by us.

Experiments 20 to 24 show how completely distinct these preparations of lactalbumin and casein are from one another. The same is shown for casein and lactoglobulin by experiments 25 to 28. Experiments 29 to 38 indicate that the alcohol soluble protein is quite distinct from casein, but that our alcohol soluble protein preparation probably contains a trace of casein, since with 5 mg. sensitizing doses 2 of 4 animals gave severe reactions with casein. Since the alcohol soluble protein is obtained from casein by washing the precipitated casein with alcohol, this admixture is to be expected.

TABLE 3

COMPARATIVE ANAPHYLACTOGENIC ACTIVITY

Protein	Minimum Fatal Sensitizing Dose, Gm.	Minimum Sensitizing Dose, Gm.	Minimal Fatal Intoxicating Dose, Intraperitoneal, Gm.	Minimal Fatal Intoxicating Dose, Intracardiac, Gm.
1. Casein	0.001	0.0001	0.010	0.0001
2. Lactalbumin	0.001	0.001	0.005	0.0001
3. Lactoglobulin	0.001	0.000,01	0.010	
4. Alcohol soluble protein	0.001	0.0001	0.010	0.0002
5. Egg albumin	0.000 001	0.000,000,1	0.0005	0.000,05
6. Egg globulin	0.000,001	.....	0.0008	
7. Edestin	0.0005	0.000.000,1	0.100	
8. Squash seed globulin	0.000,000,5	0.000,000,5	0.010	
9. Brazil nut proteose	.....	.....	0.0005	
10. Soy bean proteose	.....	.....	0.002	

Experiments 39 to 42 indicate the absence in beef serum of appreciable amounts of any protein resembling casein. Elsewhere we have reported observations showing that casein will sensitize to milk, and conversely, as has been observed by others.

The first thirteen experiments of this table indicate that lactalbumin is an active anaphylactogenic substance, thus differing from the albumin fraction of horse serum, which according to numerous observers, is very defective in anaphylactogenic properties.[47] It is much less actively anaphylactogenic than egg albumin, but corresponds closely to casein.

Experiments 14 to 25 indicate that lactalbumin and lactoglobulin are entirely distinct proteins, but that our preparations are not completely separated, for while 5 mg. sensitizing doses give little or no crossed reactions, 50 mg. sensitizing doses of either preparation will

[47] Dale and Hartley: Biochem. Jour., 1916, 10, p. 408. See Kato, Mitt. med. Fak. Univ. Tokio, 1918, 18, p. 195.

do so. As the globulin and albumin separations merely depend on the fact that the globulin comes down in a neutral saturated solution of magnesium sulphate, while the albumin is precipitated from the filtrate of this precipitation by acidification, it is remarkable to find that the separation can be made so nearly complete. The fact that

TABLE 4

LACTALBUMIN

	Sensitizing Dose, Gm.	Intoxicating Dose. Gm.	Results	Remarks
	Lactalbumin	Lactalbumin		
1	0.005	0.070	Died, 17 minutes	
2	0.005	0.070	Died, 20 minutes	
3	0.100	0.050	Severe	Note large sensitization dose
4	0.050	0.050	Died, 30 minutes	
5	0.001	0.050	Died, 20 minutes	
6	0.000,1	0.050	0	
7	0.000,01	0.050	0	
8	0.005	0.030	Died, 35 minutes	
9	0.005	0.020	Moderate	
10	0.005	0.010	Died, 70 minutes	
11	0.005	0.005	Died, 50 minutes	
12	0.005	0.000.1	Died, 20 minutes	Intracardiac
13	0.005	0.000.05	0	Intracardiac
	Lactalbumin	Lactoglobulin		
14	0.005	0.070	0	
15	0.005	0.070	0	No protection
16	0.005	0.050	0	
17	0.005	0.050	0	
18	0.100	0.050	Severe	Note large sensitization dose
19	0.050	0.050	Severe	
	Lactoglobulin	Lactalbumin		
20	0.005	0.070	Moderate	Partial protection
21	0.005	0.070	Slight	Partial protection
22	0.050	0.050	Severe	Note large sensitization dose
23	0.050	0.050	Severe	
24	0.005	0.050	0	No protection
25	0.005	0.050	0	Partial protection
	Lactalbumin	Casein		
26	0.005	0.050	0	
27	0.005	0.050	0	
	Casein	Lactalbumin		
28	0.005	0.050	Slight	
29	0.005	0.050	0	
30	0.050	0.100	Slight	Note large sensitization dose
	Lactalbumin	Alcohol soluble protein		
31	0.005	0.050	0	
32	0.005	0.050	0	
	Alcohol soluble protein	Lactalbumin		
33	0.005	0.050	0	
34	0.005	0.050	0	
	Lactalbumin	Beef serum		
35	0.005	0.5 c c	0	No protection
36	0.005	0.5 c c	Slight	
	Beef serum	Lactalbumin		
37	0.1 c c	0.070	0	Partial protection
38	0.5 c c	0.050	0	No protection

we are here dealing with distinctly different proteins is certainly demonstrated. Experiments 26 to 30 show that lactalbumin is a distinct protein, and that it can be separated from casein almost perfectly.

The complete distinction of lactalbumin from the alcohol soluble protein is established by experiments 31 to 34, while the distinction from casein has already been commented on.

That the albumin fraction of cow's milk is distinct from the protein called albumin, or any other protein, of cow serum, is shown by experiments 35 to 38. This agrees with the demonstration by Crowther

TABLE 5

LACTOGLOBULIN

	Sensitizing Dose, Gm.	Intoxicating Dose. Gm.	Results	Remarks
	Lactoglobulin	Lactoglobulin		
1	0.005	0.070	Died, 24 minutes	
2	0.005	0.070	Died, 10 minutes	
3	0.005	0.050	Died, 25 minutes	
4	0.005	0.020	Slight	
5	0.005	0.015	Severe	Nearly died
6	0.005	0.010	Severe	Died after 8 hours
7	0.005	0.010	Moderate	
8	0.005	0.005	Slight	
9	0.050	0.050	Moderate	Note large sensitization dose
10	0.050	0.050	Severe	
11	0.005	0.000,1	0	Intracardiac
12	0.005	0.000,2	Doubtful	Intracardiac
13	0.005	0.000,5	Slight	Intracardiac
14	0.001	0.050	Died, 40 minutes	
15	0.000,1	0.050	Severe	
16	0.000,01	0.050	Severe	
17	0.000,001	0.050	Slight	
	Lactoglobulin	Lactalbumin		
18	0.005	0.070	Slight	
19	0.005	0.070	Moderate	
20	0.050	0.050	Severe	Note large sensitization dose
21	0.050	0.050	Severe	
22	0.005	0.050	0	Not protected
23	0.005	0.050	0	Not protected
	Lactalbumin	Lactoglobulin		
24	0.005	0.050	0	Not protected
25	0.005	0.050	0	Not protected
26	0.100	0.050	Severe	Note large sensitization dose
27	0.050	0.050	Severe	
28	0.005	0.050	0	
29	0.005	0.050	0	
	Lactoglobulin	Casein		
30	0.005	0.050	0	Not protected
31	0.005	0.050	Slight	Not protected
	Casein	Lactoglobulin		
32	0.005	0.050	0	
33	0.005	0.050	Doubtful	
	Lactoglobulin	Alcohol soluble protein		
34	0.005	0.050	0	
35	0.005	0.050	0	
	Alcohol soluble protein	Lactoglobulin		
36	0.005	0.050	0	
37	0.005	0.050	0	
	Lactoglobulin	Beef serum, c c		
38	0.005	0.5	Severe	
39	0.005	0.5	Died, 25 minutes	
40	0.005	1.0	Died, 32 minutes	
41	0.005	1.0	Died, 42 minutes	
42	0.001	0.5	Died, 35 minutes	
43	0.001	0.5	Died, 20 minutes	
	Beef serum, c c	Lactoglobulin		
44	1.0	0.060	Slight	
45	1.0	0.040	Moderate	
46	0.5	0.050	Severe	
47	0.5	0.050	Moderate	
48	0.1	0.050	Died, 25 minutes	
49	0.1	0.050	Severe	

and Raistrick [41] of the chemical distinction between bovine lactalbumin and bovine serum albumin.

TABLE 6
ALCOHOL SOLUBLE PROTEIN

	Sensitizing Dose, Gm.	Intoxicating Dose. Gm.	Results	Remarks
	Alcohol soluble protein	Alcohol soluble protein		
1	0.005	0.050	Died, 10 minutes	
2	0.005	0.050	Died, 15 minutes	
3	0.005	0.050	Slight	
4	0.005	0.050	Died, 5 minutes	
5	0.005	0.020	Slight	
6	0.005	0.015	Died, 25 minutes	
7	0.005	0.010	Died, 25 minutes	
8	0.005	0.005	Severe	
9	0.005	0.000,1	Moderate	Intracardiac
10	0.005	0.000,2	Died, 2 minutes	Intracardiac
11	0.005	0.000,2	Died, 4 minutes	Intracardiac
12	0.001	0.100	Died, 40 minutes	
13	0.000,1	0.100	Severe	
14	0.000,01	0.100	0	
15	0.000,001	0.100	0	
	Alcohol soluble protein	Casein		
16	0.005	0.050	Moderate	
17	0.005	0.050	Severe	
18	0.001	0.050	0	
19	0.001	0.050	0	
20	0.005	0.050	Died, 10 minutes	
21	0.005	0.050	Slight	
	Casein	Alcohol soluble protein		
22	0.005	0.050	Moderate	Not protected
23	0.005	0.050	Died	
24	0.001	0.050	Slight	
25	0.001	0.050	0	
26	0.005	0.050	0	
27	0.005	0.050	Moderate	
	Alcohol soluble protein	Milk		
28	0.003	3 c c	0	Protected
29	0.003	3 c c	0	Protected
30	0.003	3 c c	Died, 20 minutes	
31	0.003	2 c c	Severe	
32	0.004	2 c c	Severe	
33	0.004	2 c c	Severe	
	Milk	Alcohol soluble protein		
34	0.4 c c	0.050	Slight	
35	0.4 c c	0.050	0	
36	0.1 c c	0.050	0	No protection
37	0.1 c c	0.050	0	
38	0.2 c c	0.050	Slight	No protection
39	0.2 c c	0.050	Died, 40 minutes	
40	1.0 c c	0.050	0	
41	1.0 c c	0.050	Slight	
	Alcohol soluble protein	Lactalbumin		
42	0.005	0.050	0	
43	0.005	0.050	0	
	Lactalbumin	Alcohol soluble protein		
44	0.005	0.050	0	
45	0.005	0.050	0	
	Alcohol soluble protein	Lactoglobulin		
46	0.005	0.050	0	
47	0.005	0.050	0	
	Lactoglobulin	Alcohol soluble protein		
48	0.005	0.050	0	
49	0.005	0.050	0	
	Alcohol soluble protein	Gliadin		
50	0.005	0.050	0	Not protected
51	0.005	0.050	0	
	Gliadin	Alcohol soluble protein		
52	0.005	0.050	0	
53	0.005	0.050	0	
	Beef serum	Alcohol soluble protein		
54	0.5 c c	0.050	0	
55	0.5 c c	0.050	0	
	Alcohol soluble protein	Beef serum		
56	0.005	0.5 c c	0	
57	0.005	0.5 c c	0	

Experiments 1 to 17 show that lactoglobulin is an active anaphylactogen. The experiments with casein and lactalbumin are the same as those in previous tables and have already been discussed. The individuality of lactoglobulin and the alcohol soluble protein of milk is established by experiments 34 to 37. Experiments 38 to 49 show that lactoglobulin differs from the other 3 protein fractions of milk, since even in 1 mg. doses it sensitizes effectively to beef serum, and also intoxicates animals sensitized with small amounts of beef serum, corroborating the results obtained with the complement-fixation reaction by Bauer and St. Engel.[26] We thus find that the earlier observations on the capacity of beef serum and cow's milk to sensitize to each other is due solely to the globulin of the milk. This fact is in gratifying agreement with the chemical observation of Crowther and Raistrick since it indicates that bovine lactoglobulin is chemically identical with the globulin fraction of beef serum, although lactalbumin and serum albumin appear to be chemically different proteins.

The observation of Heuner that colostrum is biologically more closely related to serum than later milk, harmonizes with the high globulin figure obtained in colostrum by Crowther and Raistrick. The fact that colostrum contains large amounts of globulin, that this globulin seems to be identical with serum globulin, and that the serum globulin fraction carries the antibodies of the blood, is in interesting agreement with observations that have been made which indicate that the suckling animal secures important accessions to its antibody defense during the first days of life.[48]

The previously reported observations that cow milk and bovine serum are uncertain in their capacity to sensitize to one another is explained by the presence of a very small amount of globulin in milk, corresponding immunologically to serum globulin, for it has been shown by Julian H. Lewis [49] that a small amount of one antigen injected together with another antigen in excessive amounts, may be completely or partly prevented from manifesting its antigenic activity. This probably explains the fact that in our experiments, 0.1 c c of beef serum sensitized more effectively than 1 c c.

We have discussed, in connection with the other tables, the complete differentiation of alcohol soluble protein of milk from the three other protein fractions. It is to be recalled that this protein has only recently been described by Osborne and Wakeman, and that the application of the anaphylaxis reaction played an important part in the differ-

[48] See Reymann: Jour. Immunol., 1920, 5, p. 227.
[49] Jour. Infect. Dis., 1915, 17, p. 241.

entiation from the other proteins of milk. When large quantities of freshly precipitated casein were extracted at room temperature with 50-70% ethyl alcohol, it was found that the alcohol had dissolved out a considerable quantity of protein. As hitherto the only known alcohol-soluble proteins were of vegetable origin, it was suspected that this material might be either a cleavage product or a derivative of the casein. The anaphylaxis test demonstrated that it was not a proteose or similar cleavage product, since these do not exhibit active anaphylactogenic properties as does this alcohol-soluble protein (exper. 1 to 15), while cross sensitization as well as chemical tests showed that it was not casein. Further chemical study showed that it yielded a different proportion of some of the amino acids than did casein and was, therefore, a newly recognized constituent of milk. Although resembling gliadin in solubility, it is chemically distinct from this protein and in agreement therewith yields negative results with cross sensitization tests with gliadin (exper. 50 to 53). The positive cross experiments with milk indicate that it is a constituent of milk, and not formed by manipulation during isolation (exper. 28 to 41). With such a relative excessive proportion of other proteins present in the milk, it is not to be expected that effective sensitization with milk can be obtained. The amounts of the alcohol soluble protein which give strong sensitization to milk are so small (3 to 4 mg.) that sensitization with admixed casein seems to be excluded, since less than 1 mg. doses of casein sensitize but little.

Experiments 54 to 57 indicate that beef serum does not contain the alcohol-soluble protein of milk.

SUMMARY

Cow's milk contains 4 chemically distinct proteins or protein fractions, namely, casein, lactalbumin, lactoglobulin and an alcohol-soluble protein. By means of the anaphylaxis test it can be shown that these 4 proteins are immunologically distinct. This fact furnishes another striking illustration of the dependence of immunologic specificity on chemical composition rather than biologic origin. Of these 4 proteins only one, the globulin, sensitizes to beef serum or causes reactions in animals sensitized to beef serum. This corresponds to the observation of Crowther and Raistrick that lactoglobulin and serum globulin are chemically indistinguishable. That some positive cross sensitizations may be occasionally obtained between cow's milk and beef serum is explained by the fact that the globulin constitutes a very small

part of the milk proteins. Several other protein fractions obtained in studying milk proteins were, according to anaphylaxis tests, not distinct from the 4 known proteins of milk. Our experience with milk proteins, as well as with proteins of other sources, has demonstrated that immunologic methods are a great aid and in many cases indispensable in preparing proteins in a state of purity, and may be used to furnish information concerning chemical relations of proteins from different sources.